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D-GALACTOSIDE SPECIFIC LECTINS FROM COELOMOCYTES OF THE ECHIURAN, *URECHIS UNICINCTUS*

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Abstract

Two lectins specific for D-galactosides with molecular weights of 31,000 and 34,000 (estimated by SDS-PAGE, under reduced conditions) were purified from coelomocytes of the echiuran, *Urechis unicinctus*. They were eluted together from a Bio-Gel P-100 column as a single peak with an apparent molecular weight of 35,000. They closely resembled each other in saccharide specificity, isoelectric point, and electrophoretic mobility under unreduced conditions. The hemagglutinating activity of both lectins was inhibited by a glycoconjugate fraction obtained from the Pronase-digest of coelomocytes.

Introduction

Many kinds of lectins have been identified and purified from a variety of animals including sponges (Bretting and Kabat, 1976; Müller *et al.*, 1979), snails (Hammarström and Kabat, 1969; Van der Knaap *et al.*, 1982), horseshoe crabs (Marchalonis and Edelman, 1968), insects (Komano *et al.*, 1980; Suzuki and Natori, 1983), sea urchins (Ryoyama, 1974; Sasaki and Aketa, 1981; Yamada and Aketa, 1982), fish (Teichberg *et al.*, 1975), frogs (Roberson and Barondes, 1982), chicks (Nowak *et al.*, 1977), and mammals (Hudgin *et al.*, 1974). Animal lectins are thought to participate in various physiological events such as clearance of blood components by the liver (Ashwell and Morell, 1974), adhesion of sponge cells (Müller *et al.*, 1979), differentiation of limb bud cells (Matsutani and Yamagata, 1982), infection of bacteria (Eshdat *et al.*, 1978), fertilization of *Xenopus* eggs (Wyrick *et al.*, 1974), transport and storage of sugar (Sharon and Lis, 1972), and self-defense in insects (Komano *et al.*, 1980).

The self-defense mechanisms or "immune systems" in invertebrates have been attributed mostly to coelomic fluid (hemolymph). Coelomocytes agglutinate or clump each other in response to wounding (Boolootian and Giese, 1959). The coelomic plasma contains hemagglutinins (Cushing *et al.*, 1963; Brown *et al.*, 1968; Acton and Weinheimer, 1975) or antibacterial activities (Bang and Krassner, 1958; Johnson and Chapman, 1970). However, there is little biochemical information on the echiuran coelomic fluid, although it is easily collected in large quantities (Dybas, 1981). Echiuran coelomocytes also clump if they are taken out of the body. There is no definitive evidence indicating any bactericidal activities in the coelomic fluid of echiurans. We have searched for echiuran lectins which possibly may be involved in the self-defense mechanism. This paper describes the purification and characterization of two lectins specific for D-galactoside in the coelomocytes of *Urechis unicinctus*.

Materials and Methods

Preparation of crude lectin

Echiuroid worms, *Urechis unicinctus*, were collected at Choshi, Chiba Prefecture, and at Matsuyama, Ehime Prefecture, and kept in an aquarium at 6–10°C until used.

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Purification of lectin was carried out at 4°C unless otherwise indicated. Animals were bled by cutting the body wall and the combined coelomic fluid was centrifuged at 1000 × *g* for 10 min. Coelomocytes were washed with Ca^{2+}-Mg^{2+} free sea water (500 m*M* NaCl, 9 m*M* KCl, 29 m*M* Na_2SO_4, 2 m*M* $NaHCO_3$) to eliminate germ cells, and were suspended in 10 volumes of the extraction medium; 200 m*M* lactose in medium A (0.05% Triton X-100, 10 m*M* 2-mercaptoethanol, 150 m*M* NaCl, 10 m*M* EDTA, 5 m*M* benzamidine, 10 m*M* Tris-HCl, pH 7.4). The cells were sonicated six times for 20 s at 0°C (Tomy Seiko model UR-200P, 110W) and centrifuged at 27,000 × *g* for 30 min. The supernatant was passed through a sheet of filter paper to remove floating lipids. Ammonium sulfate was gradually added to the extract to 50% saturation and the mixture was gently stirred for 4 h. The precipitate was removed by centrifugation at 27,000 × *g* for 1 h, and the concentration of ammonium sulfate was increased up to 90% saturation. The precipitate was collected by centrifugation at 27,000 × *g* for 30 min, and dissolved in 10 ml of medium A. The solution was extensively dialyzed against medium A for 48 h to eliminate lactose. The crude extract thus obtained was stored at −20°C until used.

Hemagglutination assay

Trypsinized and glutaraldehyde-fixed rabbit erythrocytes were used for standard assays. Human, swine, and bovine erythrocytes, either fresh or fixed, were also used for the test of erythrocyte specificity. Fresh erythrocytes were washed several times with medium B (10 m*M* EDTA, 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4). Trypsinized and glutaraldehyde-fixed cells were prepared according to the method of Sasaki and Aketa (1981). Since the preliminary experiment showed that Triton X-100 prevented non-specific adhesion of erythrocytes to titer plates, the hemagglutinating activity was assayed in 0.025% Triton X-100. Test solutions (25 μl) of serial 2-fold dilutions and 2% suspension of erythrocytes (25 μl) were mixed in each well of a microtiter V-plate (Cooke Engineering). The hemagglutination was scored after the plates were kept in a moisture chamber for 1 h at 20°C. The activity was expressed by titer defined as the reciprocal of the highest dilution giving positive hemagglutination. The effect of various saccharides on hemagglutination by the lectin was studied by the addition of serially diluted saccharide solutions to the hemagglutinating assay mixture containing lectin of titer 8. Inhibitory activities were expressed as minimal concentrations of saccharides to inhibit the activity of lectin. D-Thiodigalactoside (D-galactopyranosil-β-D-thiogalactopyranoside), methyl-β-D-thiogalactopyranoside, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, p-nitrophenyl-α-D-galactopyranoside, p-nitrophenyl-β-D-galactopyranoside, D-galactose, N-acetyl-D-galactosamine, methyl-β-D-xyloside, α-D-fucose, melibiose (D-glucopyranosil-β-1,6-D-galactopyranoside), fetuin, and heparin were purchased from Sigma Chemical Company. Lactose (D-glucopyranosil-β-1,4-D-galactopyranoside) was purchased from DIFCO Laboratories. L-Arabinose and D-glucose were purchased from Wako Chemicals. Glycogen and L-fucose were purchased from Katayama Chemicals.

Affinity chromatography

Lactose was conjugated to the epichlorohydrin-activated Sepharose 6B as follows. Sepharose 6B (120 ml) (Pharmacia Fine Chemicals) was washed well with distilled water (DW) and stirred in the mixture of 80 ml of 1 *M* sodium hydroxide and 10 ml of epichlorohydrin (Katayama Chemicals) for 24 h at 20°C. Activated Sepharose 6B was washed several times with DW, and was resuspended in 200 ml of 2 *M* sodium carbonate buffer (pH 10) containing 8 g of lactose. The suspension was incubated

for 3 days at 20°C with shaking. The lactose-conjugated Sepharose 6B was extensively washed with medium A and stored at 4°C until used.

About 10 ml of crude lectin extract was slowly applied to a lactose-Sepharose 6B column (2.8 × 7.5 cm). Unadsorbed materials were washed out with 150 ml of medium A. The material adsorbed to the column was eluted with medium A containing 100 m*M* lactose. Effluents were monitored at 280 nm. Each fraction of 2.5 ml was extensively dialyzed against medium A. The fractions which had hemagglutinating activity were combined and used for general lectin characterization.

Gel filtration

A column of Sephadex G-100 (1.3 × 70 cm, Pharmacia Fine Chemicals) and a column of Bio-Gel P-100 (1.3 × 52 cm, Bio Rad Laboratories) equilibrated with medium A containing 100 m*M* lactose, were used for the molecular weight estimation of native lectins. Each fraction of 0.8 ml was dialyzed against medium A to eliminate lactose and assayed for the hemagglutinating activity. In some experiments, medium A, 200 m*M* lactose in medium A, and 50 m*M* lactose-200 m*M* glucose in medium A were also used as the elution media. Bovine serum albumin (68,000), ovalbumin (43,000), deoxyribonuclease I (31,000), α-chymotrypsinogen A (25,000), and myoglobin (17,000) (Sigma Chemical Company) were used as molecular weight markers.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) using a mini-slab gel apparatus (8.4 × 9.2 cm) was performed according to Laemmli (1970) but with slight modifications. After electrophoresis, protein bands were revealed by silver staining (Wray *et al.*, 1981).

Preparative SDS-PAGE. The active fraction from the lactose-sepharose 6B column was diluted with an equal volume of sample buffer (150 m*M* Tris-HCl, pH 6.8, 0.2% SDS, 20% glycerol, 2% 2-mercaptoethanol). Aliquots of 10–20 μl were applied to the wells without heating. Electrophoresis was performed using a buffer system of 0.38 *M* glycine, 25 m*M* Tris, 0.1% SDS, and 1% 2-mercaptoethanol (pH 8.3) at 10 mA per plate. Both stacking gel (5% acrylamide) and separating gel (10% acrylamide) contained 0.1% SDS. After electrophoresis, the gel was cut into three pieces; the side pieces were used for silver staining, and the central one for purification. The central piece of the gel was sliced 1 or 2 mm thick, and each slice was extracted with 300 μl of medium A overnight at 0°C. An aliquot of each extract was reexamined for the purity by the analytical SDS-PAGE, and another aliquot was assayed for the hemagglutinating activity after extensive dialysis against medium A.

Analytical SDS-PAGE. Sample solutions were mixed with an equal volume of sample buffer (150 m*M* Tris-HCl buffer, pH 6.8, 2% SDS, 20% glycerol, 5% 2-mercaptoethanol) and the mixtures were heated at 90°C for 3 min. The samples were subjected to electrophoresis as described above.

Isoelectric focusing (IEF)

Polyacrylamide gel (5%) containing 2% Ampholine (LKB; pH 4–6) and 0.05% Triton X-100 was prepared in tubes (0.5 × 8 cm). Sodium hydroxide (20 m*M*) and phosphoric acid (10 m*M*) were used as the cathode and the anode solution, respectively. Focusing was performed at a fixed current (1 mA/tube) for 1 h, and then at a fixed voltage (400 V) for 5 h. After focusing, the gel was sliced and extracted as above.

Enzyme treatments

Lectin preparations after affinity chromatography were incubated with one of the following enzymes at its optimal pH at 25°C for various periods; trypsin (0.1 mg/ml, Sigma Chemical Company), Pronase P (0.1 mg/ml, Kaken Chemicals), protease from *Streptomyces griseus* (0.1 mg/ml, Sigma Chemical Company), α-chymotrypsin (0.1 mg/ml, Sigma Chemical Company), alkaline phosphatase (50 units/ml, Boehringer Mannheim), and glycosidase mixture from *Turbo cornutus* (0.1 mg/ml, Seikagaku Kogyo). After enzyme treatment, remaining activities were assayed at 4°C. For control experiments, enzyme solutions were replaced with the same volume of medium A.

Pronase digestion of coelomocytes

The residues after lectin extraction of coelomocytes were resuspended in 100 ml of DW and dialyzed against 2 liters of DW with 3 changes for 3 days. The dialysate was mixed with 100 ml of 0.1 *M* borate buffer (pH 8.0) containing 80 mg of Pronase P, 10 m*M* $CaCl_2$, and 0.02% sodium azide. After addition of small volume of toluene, the mixture was incubated at 37°C for 48 h with gentle shaking. The digestion by Pronase was stopped by adding trichloroacetic acid to a final concentration of 10% (w/v). The supernatant obtained after centrifugation was neutralized with 10 *N* sodium hydroxide, and was concentrated to 25 ml with a rotary evaporater at 30°C. After centrifugation of the concentrates at 27,000 $\times$ *g* for 30 min, 5 ml of the supernatant was fractionated with a Sephadex G-25 column (medium, 2.7 $\times$ 87 cm), equilibrated and eluted with 50 m*M* pyridine acetate buffer, pH 5.2. The break through fractions were concentrated with a rotary evaporater. The concentrate was dialyzed against medium A and assayed for the inhibition of the hemagglutinating activity.

Heat stability test and chemical analysis

The lectins in medium A (20 μg protein/ml) were incubated at 20, 40, and 60°C. At various time intervals, aliquots were assayed for the hemagglutinating activity at 4°C.

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard. Saccharide concentration was assayed by the phenol-sulfuric acid method (Dubois *et al.,* 1956), using glucose as a standard.

RESULTS

Extraction and purification of the lectins

A high hemagglutinating activity was detected only when coelomocytes were disrupted, while it was not detected in the coelomic plasma or in the suspension of intact coelomocytes. The activity in the cells was effectively solubilized by 100 m*M* lactose in the medium. After extraction with this medium, the activity was not detectable in the residue, indicating the apparent solubilization of 100% activity. In the absence of lactose, however, only 3% of the activity was solubilized.

The activities solubilized in the absence and in the presence of lactose differed from each other in some properties. The activity solubilized by the lactose-free medium was precipitated by ammonium sulfate at 50% saturation, and was not adsorbed to the lactose-Sepharose 6B column. On the contrary, most of the activity solubilized by the medium containing lactose precipitated between 50–90% saturation of ammonium sulfate and was readily adsorbed to the lactose-Sepharose 6B column. The activity adsorbed to the column was quantitatively eluted by 100 m*M* lactose as

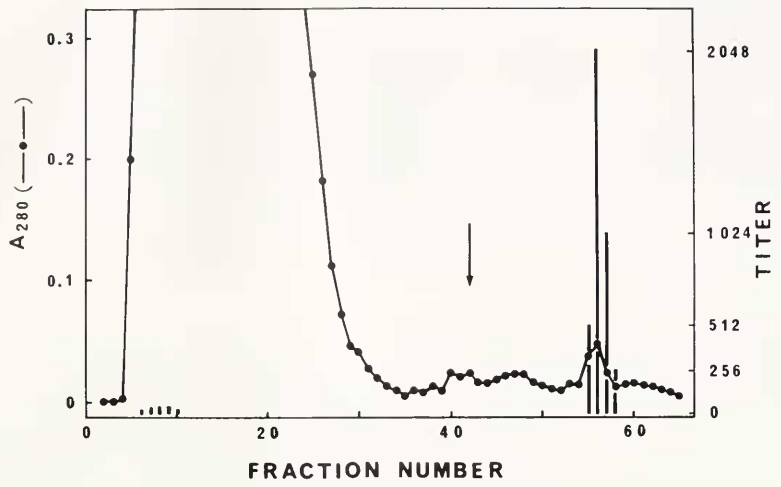

FIGURE 1. Affinity chromatography of *Urechis* lectins. Crude lectin extract obtained by ammonium sulfate precipitation was applied to a lactose-Sepharose 6B column (2.7 × 7.5 cm). The column was washed with medium A until the absorption at 280 nm became negligible and then the eluting medium was changed to 100 m*M* lactose in medium A at the point shown by the arrow. The hemagglutinating activity was eluted at a small peak of A_{280}.

shown in Figure 1. The lectin was purified 1350-fold with the yield of 23% by the procedure as summarized in Table 1.

SDS-PAGE in the presence of 2-mercaptoethanol of the lectin preparation showed two bands corresponding to the molecular weights of 31,000 and 34,000. The two components were isolated by preparative SDS-PAGE (Figs. 2, 3), but they were not separable if the concentration of SDS was less than 0.02%. Both of them retained the hemagglutinating activity (Fig. 2), and were susceptible to thiodigalactoside, lactose, and galactose.

Molecular weights of the lectins

Molecular weights of the lectins were estimated to be 31,000 and 34,000 by SDS-PAGE. However, this difference in the molecular weights was not revealed by other methods. Both lectins showed the same molecular weight of 31,000 by SDS-PAGE in the absence of 2-mercaptoethanol (Fig. 3), 26,000 by gel filtration using Sephadex

TABLE I

Purification of the lectins from the coelomocytes of Urechis unicinctus

Step	Titer	Protein (mg/ml)	Volume (ml)	Sp. act.**	Total activity	Recovery (%)
Extraction	128	3.36	97	38	12,416	100
50–90% AS ppt*	1024	3.92	10	261	10,240	82
Lactose-Sepharose 6B	1024	0.02	2.8	51,200	2,867	23

* Ammonium sulfate precipitation.
** Specific activity; Titer/mg of protein.

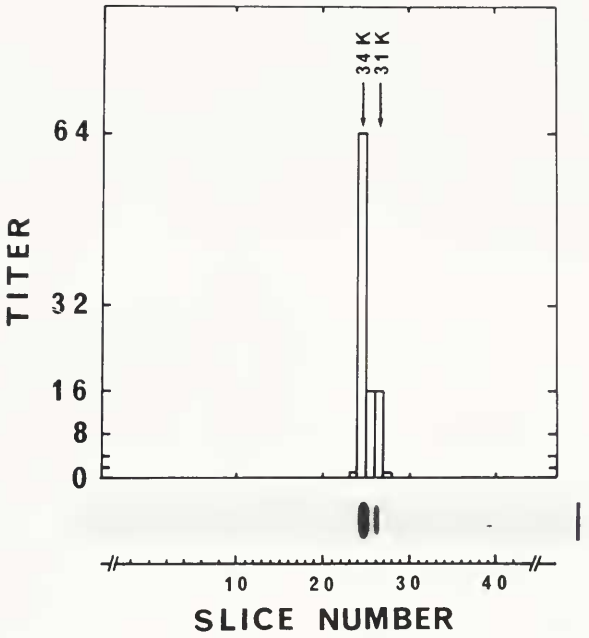

FIGURE 2. Distribution of the hemagglutinating activity after preparative SDS-PAGE. After preparative SDS-PAGE, the gel slice was extracted with medium A overnight at 0°C. The extracts were assayed for the hemagglutinating activity after extensive dialysis.

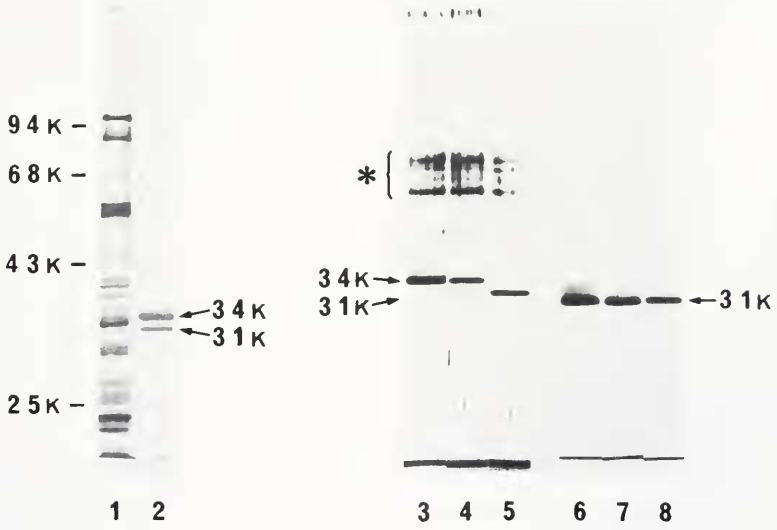

FIGURE 3. Purification of 31,000 and 34,000 lectins by preparative SDS-PAGE. SDS-PAGE (under reduced (*1–5*) and unreduced (*6–8*) conditions) of lectins. *1.* Flow through fraction of affinity chromatography. *2.* Lectin fraction after affinity chromatography. *3, 6.* The extract of slice No. 25 in Figure 2. *4, 7.* The extract of slice No. 26. *5, 8.* The extract of slice No. 27. Molecular weights of standards are indicated on the left of photographs. Bands indicated by asterisk appeared in the presence of 2-mercaptoethanol presumably by artifact as reported by Tasheva and Dessev (1983).

G-100 in the presence of 100 m*M* lactose, and 35,000 by gel filtration using Bio-Gel P-100 in the presence or absence of 100 m*M* lactose (Fig. 4). The elution profiles of the lectins from Sephadex G-100 was not modified by increasing the lactose concentration or by adding 200 m*M* glucose in the elution buffer. In the absence of lactose, both lectins were eluted much later as a single peak from Sephadex G-100 column. The fractions in the peak of activity from either of the columns were shown by SDS-PAGE to comprise 31,000 and 34,000 lectins.

Characterization of the lectins

Preparations after affinity chromatography were used for general characterization of the lectins because of a limited amount of further purified ones. *Urechis* lectin agglutinated rabbit, human (either type A, B or O), bovine, and swine erythrocytes (Table II). Rabbit erythrocytes were most readily agglutinated by the lectin. The agglutinability was increased by trypsin digestion of erythrocytes, but was not changed by fixation with glutaraldehyde. Therefore, trypsinized and glutaraldehyde-fixed rabbit erythrocytes were used for the standard assays.

Saccharide specificity of the lectins is summarized in Table III. The hemagglutinating activity of the lectins was inhibited by various D-galactosides. Thiodigalactoside was the most potent inhibitor followed by lactose and p-nitrophenyl-β-D-galactopyranoside. Anomeric configuration had a slight effect on inhibition; β-D-galactosides were more effective than α-D-galactosides. Fetuin, having the terminal sialic acids and the penultimate galactose, did not inhibit the lectin activity, while asialofetuin markedly inhibited the activity. A glycoconjugate fraction from the Pronase-digest of coelomocytes also inhibited the lectin. Glucose was a saccharide component only detected in the glycoconjugate by gas-liquid chromatography. Galactose content seemed to be very little, if any (data not shown).

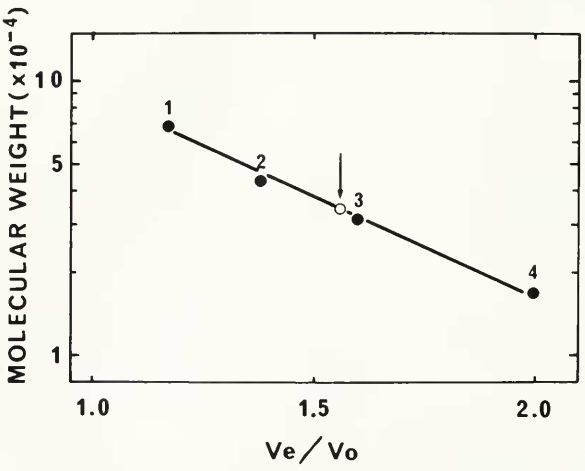

FIGURE 4. Estimation of molecular weight of *Urechis* lectins by a Bio-Gel P-100 column. The active fraction from affinity chromatography was subjected to gel filtration using a Bio-Gel P-100 column (1.3 × 52 cm). The lectins were eluted as a single peak corresponding to the molecular weight of 35,000 either in the absence or presence of 100 m*M* lactose. Bovine serum albumin (1), ovalbumin (2), deoxyribonuclease I (3), and myoglobin (4) were used as the molecular weight markers.

TABLE II

Erythrocyte specificity of Urechis *lectin*

Erythrocytes		Treatment	Titer
Human	A	none	8
	B	none	4
	O	none	4
Rabbit		none	512
		GA-fixed*	128
		trypsinized**	2048
		trypsinized & GA-fixed	2048
Swine		none	4
		GA-fixed	4
		trypsinized	4
Bovine		GA-fixed	2

The lectin preparation after affinity chromatography was used.
* Glutaraldehyde-fixed erythrocytes.
** Trypsinized erythrocytes.

TABLE III

Saccharide specificity of Urechis *lectin*

Saccharides	Minimal inhibitory concentration (m*M*)
D-Thiodigalactoside	0.05
Lactose	0.10
p-Nitrophenyl-β-D-galactopyranoside	0.10
Methyl-β-D-galactopyranoside	0.20
Methyl-β-D-thiogalactoside	0.39
p-Nitrophenyl-α-D-galactopyranoside	0.78
Methyl-α-D-galactopyranoside	0.78
D-Galactose	0.78
Melibiose	0.78
α-D-Fucose	1.56
L-Arabinose	6.25
N-Acetyl-galactosamine	6.25
Methyl-β-D-xyloside	>50
L-Fucose	>50
D-Glucose	>50

Glycoconjugates	Minimal inhibitory concentration (mg/ml)
Asialofetuin	0.21
Pronase-digest of coelomocytes	0.35*
Fetuin	>3.75
Heparin	>2.5
Glycogen	>8.99

* mg hexose/ml.
Lectin preparation after affinity chromatography was used.
Lectin titer was previously adjusted to 8.

The activity of the lectin was diminished by heating at 60°C for 5 min. No significant loss of the activity was observed after storage in medium A for 3 months at −20°C. No appreciable change in the activity was observed in the absence or presence of 2-mercaptoethanol up to 140 m*M*. However, the activity was preserved much better in the presence than in the absence of 10 m*M* 2-mercaptoethanol at 4°C. The lectins seemed to not require divalent cations for their activity. Most of the activity was lost after several cycles of freezing and thawing. The activity was completely destroyed by proteases (trypsin, Pronase P, α-chymotrypsin, and bacterial protease) but was resistant to glycosidase mixture or alkaline phosphatase. The lectins contained sugars (8.4% of protein). No significant change in the molecular weight was observed even after the treatment of the lectins with glycosidase or alkaline phosphatase. The two lectins were focused by IEF to a single peak of activity with a pI of 5.0–5.5.

Discussion

Most invertebrate lectins have been isolated from hemolymph before or after coelomocyte clumping (Marchalonis and Edelman, 1968; Anderson, 1980; Komano *et al.,* 1980). To prepare hemolymph, little attention has been paid to the integrity of coelomocytes. Thus the source of lectins remains problematical. In *Urechis,* no hemagglutinating activity was detected in the coelomic plasma if the coelomocytes were carefully removed without clumping. Further, a high hemagglutinating activity was detected in the homogenate of washed coelomocytes. From these observations, it is assumed that *Urechis* lectins are localized only in the coelomocytes. Similar distribution of lectin has been reported in lobster (Cornick and Stewart, 1973).

Two lectins were not separable by gel filtration, affinity chromatography, IEF, or PAGE in the absence of SDS or 2-mercaptoethanol. With the exception of molecular size, this may suggest a mutual affinity and/or close similarity in their physicochemical properties. The apparent molecular weight of 34,000 lectin was decreased to 31,000 by SDS-PAGE in the absence of 2-mercaptoethanol. This indicates the presence of disulfide bond(s) in the 34,000 lectin. Since the difference in apparent molecular weights between reduced and unreduced form was rather small, the cystein residues forming disulfide bond(s) seem to locate rather closely along the peptide chain.

The lectins have an affinity to dextran (Sephadex). This affinity seems to be cancelled mostly, but not completely, by the addition of lactose. Lectins may not have an affinity to polyacrylamide gels, since they were eluted from a Bio-Gel P-100 column as a single peak corresponding to the molecular weight of 35,000 in the presence or absence of lactose. The estimated molecular weight was close to those obtained by SDS-PAGE. This suggests that these lectins are monomeric and that the affinity between the lectins is not significant.

Most of the lectin in the coelomocytes was extracted by inclusion of lactose in the extraction medium as reported with chick muscle lectin (Nowak *et al.,* 1977). Only a small portion of the hemagglutinating activity was extractable by lactose-free medium. *Urechis* lectin extracted with lactose-free medium was not adsorbed to the affinity column and was recovered in the break through fractions from a Sephadex G-100 column (data not shown). The lectins seem to bind some macromolecules, which are removed from the lectins by the addition of lactose. One of the candidates for such a putative macromolecule is a glucose-rich glycoconjugate, obtained from coelomocytes after the Pronase digestion. Though the glycoconjugate inhibited the hemagglutinating activity of the lectins, galactose was not detected appreciably in it. Susceptibility of the lectins to the glucose-rich glycoconjugate and their affinity to dextran imply a role for the lectins in the storage of saccharides as suggested by

Sharon and Lis (1972). Ochi (1966) found many glycogen granules in the coelomocytes of *Urechis unicinctus* by histochemical and electron microscopic observations. It is presently uncertain whether the glucose-rich endogenous inhibitor is glycogen. Commercial glycogen and glucose were not inhibitory against the lectins.

We first expected that *Urechis* lectins might participate in the clumping of coelomocytes as a part of self-defense mechanism, but we could not observe any inhibition of the clumping by lactose. Furthermore, the *Urechis* lectins did not agglutinate the glutaraldehyde-fixed coelomocytes which were readily agglutinated by *Ricinus communis* agglutinin. Physiological roles of *Urechis* lectins await further investigation.

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